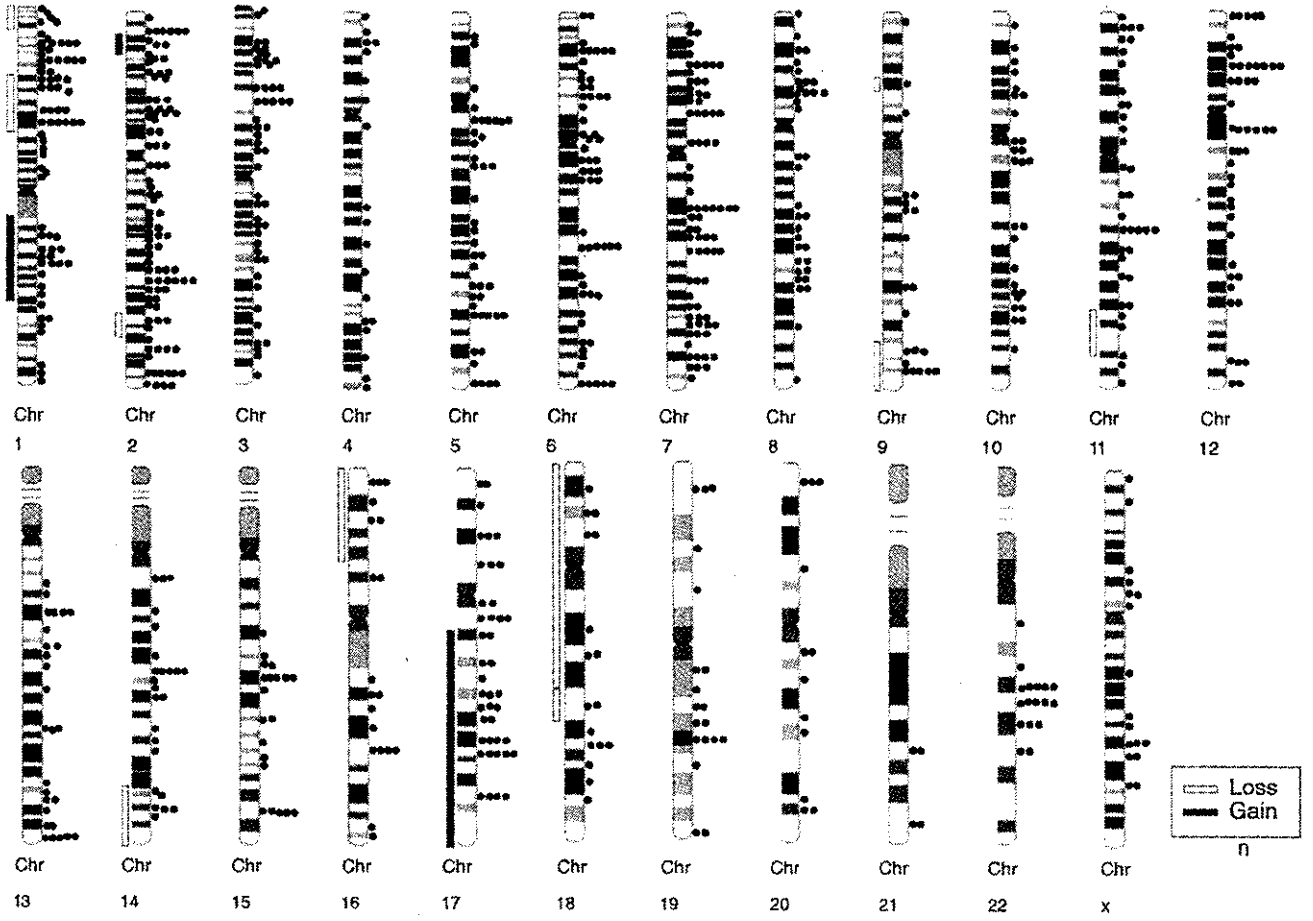

Fig. 3. Chromosomal mapping of the genes differentially expressed between favorable and unfavorable subsets of neuroblastoma. The gray and black lines indicate the regions of loss of heterozygosity (LOH) and those of chromosomal gain, respectively.

development as well as its aberrations in cancers such as NBL. Furthermore, the NBL cDNA microarray should be important for the clinical use to develop it as a diagnostic tool. The comparative and parallel studies between the results obtained from the cDNA microarray analysis and the array comparative genomic hybridization (array CGH) [57] might also be very helpful to identify the genes and to understand the molecular biology of NBL.

6. Developmental clock and the genes of neuroblastoma

Our NBL cDNA project has provided us with tremendous information about the genes expressed in different subsets with characteristic biology. Though the project is still ongoing, a temporary view so far obtained suggests the presence of a kind of rule in the expression patterns of the subset-specific genes. Fig. 4 shows the groups of genes expressed along the time axis of sympathetic neuron development. During the early stages of development, many transcription factors and their regulators may play important roles in deciding the direction of differentiation as well as in regulating cell growth and survival of neural crest-derived cells. It is interesting that many genes highly expressed in unfavorable NBLs contain transcription factors and the components of their complexes. They involve MYCN and Id family transcription factors that link to the regulation of Rb and p53 and regulate cell growth and apoptosis. The basic helix-loop-helix transcription factor, hASH1, is constitutively activated in NBL, and, by collaborating with Phox2a and Phox2b, it may regulate the arrest of

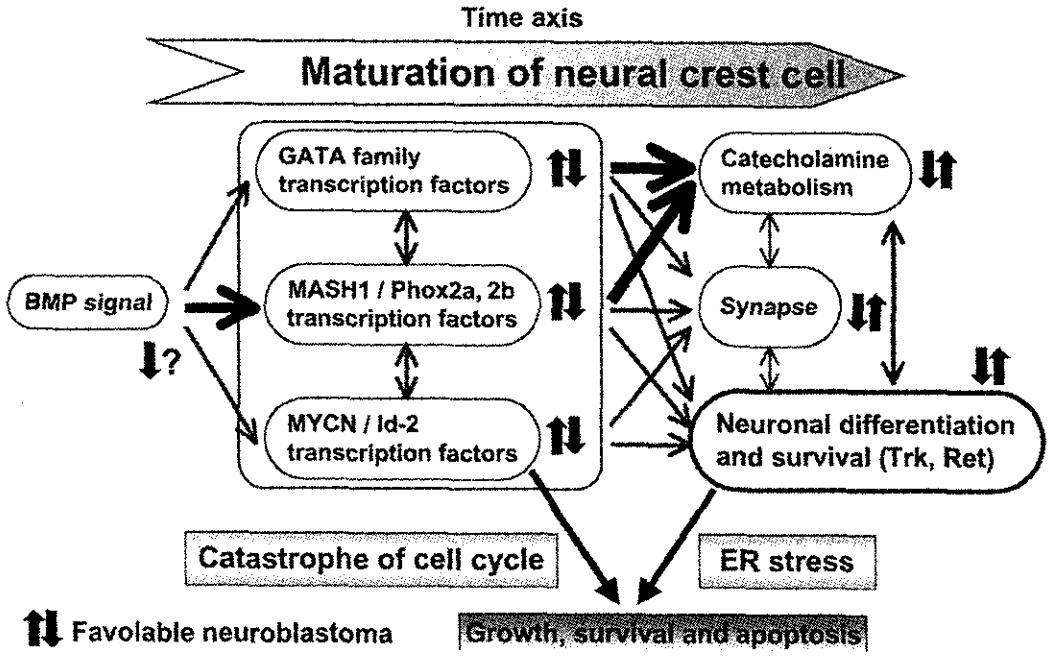

Fig. 4. The developmental time axis and the gene expression cascade during maturation of the neural crest cells. Many transcription factors are up-regulated in the unfavorable neuroblastoma, whereas the genes related to the terminal differentiation of neuron are up-regulated in the favorable neuroblastoma. ER: endoplasmic reticulum.

differentiation in unfavorable NBLs (T. Kuno et al., unpublished data). Our NBL cDNA project has also revealed that there may exist a neuronal cassette of GATA transcription factor complex that controls growth and differentiation of sympathetic progenitor cells. Some molecules in this complex are up-regulated in unfavorable NBLs (M. Aoyama et al., manuscript in preparation). Thus, many important components in the transcriptional regulators appear to be highly expressed in unfavorable NBLs and function to regulate the tumor cell growth or the status of de-differentiation.

On the other hand, a remarkable number of the genes expressed at high levels in favorable NBLs seem to encode the molecules that are necessary to maintain the neuronal function of the matured neuronal cells. They may be needed to maintain catecholamine metabolism, synapse formation, neuronal cell survival, etc. We have also identified many genes related to the ubiquitin-proteasome pathway and heat shock proteins in favorable NBLs, suggesting that they might be involved in induction of apoptosis triggered by endoplasmic reticulum (ER) stress [58].

Thus, our NBL cDNA project which is still on-going has clearly demonstrated that favorable NBL arrests its differentiation at late stage of neural development, whereas unfavorable NBL arrests at the early and immature stage.

7. From comprehensive to functional genomics of neuroblastoma aiming at the drug discovery

All genes and their products are no doubt necessary to accomplish embryonal differentiation and to maintain homeostasis of the whole human body. However, aberration of even one gene can cause disease or cancer, albeit many cancers including NBL have multiple genetic abnormalities. Therefore, we need to try to select the truly important genes that

affect the genetics and biology of NBL as key regulators.

During the functional analyses of the novel genes we selected for the last three years, several genes have been reported as important regulators especially in the neuronal system. They include Nogo (Nbla00271) [59], a negative regulator of axonal guidance, FOG2 (Nbla03139) [60], a cofactor of the GATA transcription factor complex, small GTPase RAB6B (Nbla00086) [61], Mlt1 (Nbla00106) [62], homeotic regulator homolog MAB21 (Nbla00126) [63], GTP-binding protein RAB3C (Nbla00494) [64], neurexophilin (Nxph1, Nbla00697) [65], RAB3 efffector protein RIM1 (Nbla00761) [66], cell recognition molecule Caspr2 (Nbla00831) [67], endothelin converting enzyme-like 1 (ECEL1, Nbla03145) [68], doublecortin- and calmodulin kinase-like 1 (DCAMKL1, Nbla10919) [69] and aczonin (Nbla11270) [70]. Our current analyses of the individual genes selected also suggest that the present cDNA resources derived from the primary NBLs contain many novel genes involved in NBL, neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, and other diseases. The progress of the functional genomics of the NBL genes may lead to the discovery of the molecular targets for the therapeutic strategy.

8. Conclusion

The enigmatic tumor, neuroblastoma, is changing not to be enigmatic, because the molecular bases are now rapidly being unveiled. Indeed, the clinical and biological enigma of NBL may suggest a tight link between the cancer and the development of neural crest cells from which NBL is derived. The comprehensive genomics and future proteomics approaches may accelerate further understanding of the biology of NBL which in turn helps to clarify the molecular mechanism of normal neural development. It is important and interesting that the key regulators during the normal neural development are usually the targets of the NBL genesis. We need to intensively search for those target genes which could also be important for the drug discovery leading to the cure of the patients.

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LETTER TO THE EDITOR
Extraovarian Primary Peritoneal Carcinoma in a Child†

Key words: extraovarian primary peritoneal carcinoma; ovarian cancer; CA125; childhood cancer

Extraovarian primary peritoneal carcinoma (EOPPC) was first described in 1959 by Swerdlow [1]. It is an adenocarcinoma that develops from the peritoneum lining the pelvis and abdomen and is characterized by abdominal carcinomatosis, uninvolved or minimally involved ovaries, and no identifiable primary tumor. It accounts for ~10% of cases with a presumed diagnosis of ovarian cancer [2]. It is similar to ovarian serous carcinoma with respect to clinical presentation, histologic appearance, and response to chemotherapy [3]. The median age of recorded cases is 57–66 years [3] and there has been no prior report in a child. Our experience in such a case is of interest.

Our patient was 13-year-old girl, who was admitted to St. Luke's International Hospital, Tokyo, Japan, because of a huge abdominal mass. Her chief complaint was abdominal pain and constipation. A hard mass, about 5 cm × 6 cm in size, was palpable in the lower abdomen upon physical examination. Thyroid gland was not enlarged. Laboratory data were within normal limits except for CA125, which was 453.8 U/ml. Abdominal CT and MRI revealed a large, solid mass that occupied the pelvic cavity (Fig. 1), and no other tumor lesions were detected. The diagnosis before operation was a primary ovarian tumor. An open biopsy was performed and the histologic diagnosis was serous adenocarcinoma (Fig. 2). The size of the tumor did not decrease at all after three course of chemotherapy consisting of vindesine, doxorubicin, and fluorouracil (5-FU). Surgery performed 3 months after the biopsy removed the tumor together with the uterus, adnexa, and a part of the rectum and the sigmoid colon because the tumor directly invaded the uterus and large bowel. An endometrial cyst, 5 cm in diameter and attached to the neoplasm was also resected. The excised mass measured 9.0 cm × 6.0 cm × 4.5 cm (Fig. 3, arrowheads). The left ovary was 4.0 cm × 1.5 cm × 1.0 cm (Fig. 3, arrow) and no tumor cells were seen in the ovarian tissue. The size of the right ovary was 4.5 cm × 4.0 cm × 2.5 cm. There were adenocarcinoma components within the ovarian stroma. Since the ovary was not in continuity with the tumor, it was considered to be a metastatic lesion. Both fallopian tubes were normal. From the operative findings and histologic examination, it appeared that tumor originated from extraovarian tissue. It was, therefore, diagnosed as an EOPPC. Seventeen months after the operation, a vaginal recurrence was noted. Colpectomy was carried out and radiation therapy (total 50 Gy) was added. Six months after radiation, the tumor recurred in the pelvic cavity and abdominal wall. Management then became palliative to control pain by morphine sulfate, instead of aggressive chemotherapy and repeat surgery. She died 50 months after the diagnosis.

Most malignant ovarian tumors in adults are epithelial in type, but in children and adolescents, 60% are of germ cell origin [4]. Ovarian carcinoma is rare, and extraovarian carcinoma is extremely rare in children. EOPPC should nonetheless be added to the differential diagnosis when a tumor occupying the abdominal cavity and considered of ovarian origin is encountered in a child.

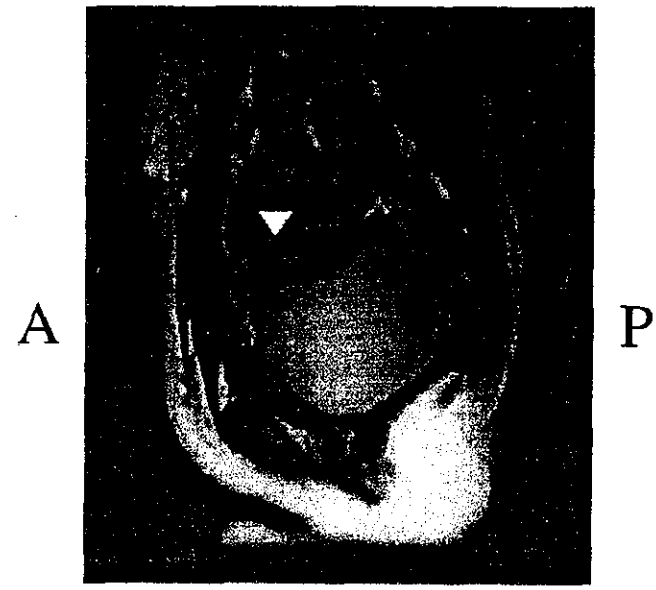

Fig. 1. Sagittal T_2-weighted abdominal MRI. A large, solid tumor, 10 cm × 10 cm × 8 cm in size, occupied the pelvic cavity. A small, cystic mass lesion lay above the solid tumor (arrowhead). This mass turned out to be an endometrial cyst at surgery. A: anterior, P: posterior.

The differential diagnosis should also include desmoplastic small round cell tumor in which elevated serum CA125 has been reported [5,6]. However, the pathologic findings of our case were completely different. No psammoma bodies or papillary and glandular structures are to be found in desmoplastic small round cell tumors.

It has been reported that levels of the tumor marker CA125 were elevated (>35 U/ml) in most of the EOPPC

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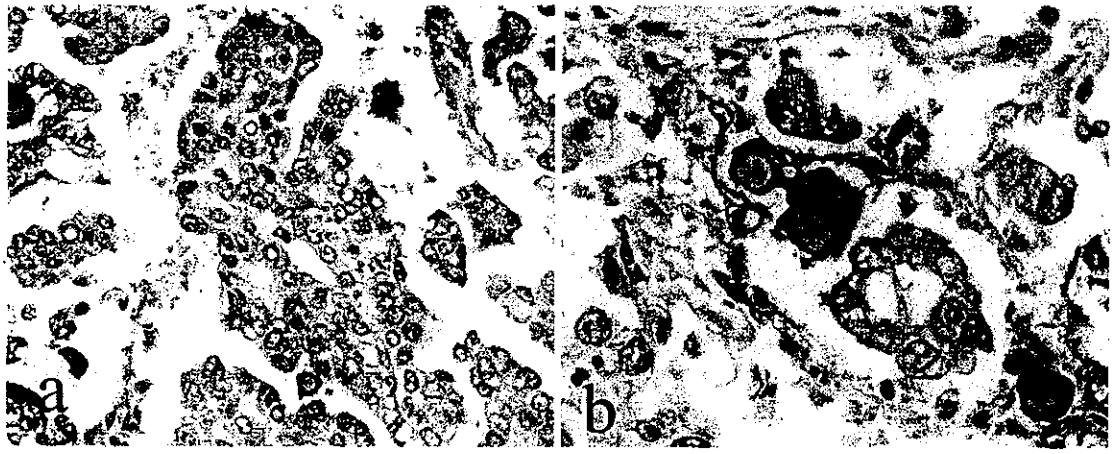

Fig. 2. Hematoxylin and eosin stain. a: The tumor cells with nuclear atypia showed papillary growth (original magnification × 200). b: Psammoma bodies were seen in the tumor tissue (original magnification × 400).

patients [7,8]. In our patient, the CA125 levels correlated with the clinical status of the disease and response to therapy. This finding has also been reported in reference [7]. It is important to measure CA125 not only during chemotherapy but also thereafter.

The response to chemotherapy and the prognosis of EOPPC is said to be similar to serous ovarian carcinoma [3]. However, there have been some reports that the prognosis for EOPPC is worse than for ovarian serous cancers with comparable peritoneal spread [2,8]. Reported median survival durations vary between 7.0 and 27.8 months and 5-year survival rates range from 0 to 26.5% among EOPPC patients [3]. Our patient suffered repeated relapses in spite of tumor excision, chemotherapy, and radiation therapy. We chose "quality of life" management after chemotherapy and surgery failed to cure the tumor, so that the patient could go back to high school and travel abroad with her family.

In summary, we here report the first case of EOPPC in a child. Aggressive combined modality therapy failed to cure the girl.

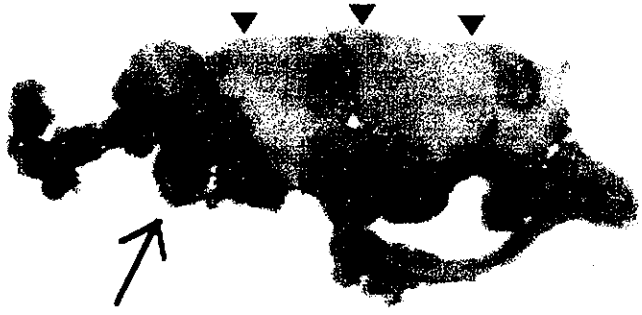

Fig. 3. Gross appearance of the resected tumor and ovaries. The tumor (arrowheads) was solid and its color was yellow and white with degeneration and necrosis. It measured 9.0 cm × 6.0 cm × 4.5 cm. The left ovary (arrow) was 4.0 cm × 1.5 cm × 1.0 cm in size. No tumor cells were seen in the ovarian tissue.

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Original Article

Tumor angiogenesis in the bone marrow of multiple myeloma patients and its alteration by thalidomide treatment

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Angiogenesis in solid tumors is important to tumor growth, invasion and metastasis. Recently, it has been suggested that angiogenesis plays a certain role in the development of hematopoietic malignancies, including leukemia and multiple myeloma. We evaluated tumor angiogenesis in the bone marrow (BM) of multiple myeloma (MM) patients by calculating microvessel density (MVD) in needle-biopsy specimens obtained from 51 cases of untreated MM or monoclonal gammopathy of undetermined significance (MGUS). The MVD in the BM of donors for transplantation and patients with non-hematological diseases was calculated as a control. There was an obvious increase in MVD in the BM of MM patients, and the MVD correlated with the grade of myeloma cell invasion of the BM in the untreated MM cases. It was recently reported that thalidomide might be effective for the treatment of MM. We assessed the effect of thalidomide on angiogenesis in BM treatment of 11 patients with refractory MM. The concentration of M-protein in the serum or urine of seven of the 11 patients was reduced by at least 30% after thalidomide treatment, and MVD in the BM decreased in three of these seven cases in response to thalidomide. Increased plasma concentrations of basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) were observed in all 11 cases before thalidomide administration and both levels were reduced after treatment with thalidomide. Augmented angiogenesis in the bone marrow of MM patients was confirmed in the present study. It seems that thalidomide is effective in the treatment of MM through the impairment of angiogenesis by decreasing FGF-2 and VEGF production. This is the first report on pathological evidence in the bone marrow of MM before and after thalidomide treatment, in Japan.

Key words: angiogenesis, CD34, fibroblast growth factor, multiple myeloma, thalidomide, vascular endothelial growth factor

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Multiple myeloma (MM) is a plasma cell neoplasm that originates in the bone marrow and involves the entire skeleton. The myeloma cells synthesize large amounts of complete and/or incomplete immunoglobulins, which may cause a hyperviscosity syndrome, amyloidosis, and renal failure. Multiple myeloma accounts for approximately 10% of all hematological malignancies. Chemotherapy with melphalan and/or dexamethasone is effective in some cases but relapses often occur, and relapsed MM might become refractory to conventional chemotherapy.[1]

Angiogenesis is indispensable to growth, invasion and metastasis by solid tumors and occurs in other diseases, including rheumatoid arthritis, psoriasis, scleroderma and diabetic retinopathy.[2–4] Angiogenesis has been postulated to be regulated by a balance between certain angiogenic and antiangiogenic factors. Angiogenic factors include soluble factors, such as vascular endothelial growth factor (VEGF), acid and basic fibroblast growth factor (aFGF and bFGF or FGF-2), angiopoietin-1, hepatocyte growth factor (HGF) and interleukin-8 (IL-8), and adhesion molecules, such as integrins.[5–8] Vascular endothelial growth factor is considered an important vasculogenic mediator of embryonic and postnatal angiogenesis that functions in the promotion of endothelial cell growth and/or inhibition of apoptosis.[9] Elevated VEGF concentrations have been reported in several types of metastatic cancers, suggesting that it plays a role in cancer progression.[10] FGF-2 has been reported to be a potent stimulator of angiogenesis *in vitro* and is found in the serum and/or urine of patients with several types of cancer, including leukemias.[11,12] Angiogenesis has recently been reported in hematological malignancies, such as leukemia and myelodysplastic syndromes.[11,13–18] Vacca *et al.* reported finding positive correlations between increased bone marrow microvessel density (MVD) and both the plasma-cell labeling index and disease activity in MM patients.[19] The increased plasma or serum concentrations of FGF-2 and VEGF has been observed not only in solid tumors, such as prostate

cancer and renal cancers, but also in hematological malignancies, such as acute lymphocytic leukemia, acute myeloleukemia, chronic myeloleukemia, and also MM.[11,13,18] Thus, it is critical to determine whether antiangiogenetic therapy will be useful for the treatment of hematological malignancies.

Thalidomide was introduced in the 1950s as a sedative, but was withdrawn from the market in the 1960s because of its teratogenicity.[20] Recently, thalidomide has been found to be effective against erythema nodosum leprosum,[21] graft-versus-host disease[22] and Crohn's disease.[23] An antiangiogenic function of thalidomide in a rabbit cornea micropocket assay was reported by D'Amato *et al.* in 1994.[24] Rajkumar *et al.* reported that tumor angiogenesis occurs in the bone marrow of MM patients and that thalidomide therapy improved the MM in some previously treated myeloma patients.[25]

We recently reported that the elevated level of plasma FGF-2 in MM patients correlated with increased disease activity.[26] Furthermore, we showed that thalidomide was effective for the treatment of refractory MM in Japan.[27] Hence, in the present study, we assessed the MVD of the bone marrow of untreated MM patients to confirm tumor angiogenesis in MM. We also investigated the effectiveness of thalidomide treatment for refractory MM patients and whether thalidomide administration altered tumor angiogenesis.

MATERIALS AND METHODS

Patients

To study angiogenesis in MM, we evaluated 51 bone marrow biopsy specimens from 51 untreated MM patients at Keio University Hospital. The clinical characteristics of the patients are shown in Table 1. The 51 patients consisted of 28 males and 23 females, and their median age was 62 years. Forty-two cases fulfilled the diagnostic criteria for MM of the South West Oncology Group (SWOG), and the other nine cases were diagnosed with a monoclonal gammopathy of undetermined significance (MGUS) based on the SWOG criteria.[28] The major subclass of immunoglobulin heavy chains was IgG (32 out of 51 cases). We used six bone marrow specimens, two from transplantation donors and four obtained at autopsy from patients with non-hematological diseases (pneumonia in two cases and cancer without bone marrow involvement in the other two cases).

To evaluate the effect of thalidomide on angiogenesis, we analyzed bone marrow biopsy specimens before and after thalidomide administration to 11 patients with refractory MM (six females and five males) who had been treated with conventional chemotherapy, such as vincristine, melpharan, prednisolone and dexamethasone (Table 2). Two patients also received peripheral blood stem cell transplantation and Interferon alpha (IFNα) before being treated with thalidomide. The disease of all 11 patients was categorized as Durie-Salmon stage 3 before treatment with thalidomide.[29]

Table 1 Clinical parameters of untreated patients

Number of patients	51
Average age (years)	62 (range, 37–88)
Male/Female	28/23
Subclass of Ig	
IgG	32
IgA	11
IgD	2
BJP	5
Nonsecretory	1
Stage (Durie-Salmon)	
1	15
2	6
3	21
MGUS	9

BJP, Bence Jones proteins; Ig, immunoglobulin; MGUS, monoclonal gammopathies of undetermined significance.

Immunohistochemistry

Sections of paraffin-embedded bone marrow specimens were stained by a standard indirect immunohistochemical technique with anti-CD34 mouse monoclonal antibody (Novocastra, Newcastle, UK) to highlight endothelial cells. The myeloma cells in the bone marrow were stained with antihuman plasma cell mouse monoclonal antibody (VS38c; DAKO, Glostrup, Denmark). The bone marrow specimens of the patients treated with thalidomide were also stained with anti-FGF-2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-FGF Receptor 1 rabbit polyclonal antibody generated by immunizing a rabbit with a synthetic oligopeptide of human FGF Receptor 1.[30]

Measurement of bone marrow microvessel density and the grading of myeloma cell invasion

All bone marrow biopsy specimens were evaluated for cellularity by light microscopy with a 10× power objective lens. Five areas with high cellularity were randomly selected and examined with an 80× power objective lens. Five fields were taken, with each field representing an area of 0.108 mm^2. Individual microvessels (stained brown by immunohistochemistry anti-CD34 antibody) were counted in each fields and their density was calculated.

The grade of myeloma cell invasion in the bone marrow was determined by both hematoxylin–eosin (HE) staining and immunohistochemistry with anti-VS38c antibody. Invasion was graded as 'mild' if myeloma cells accounted for less

Table 2 Summary of patients treated with thalidomide

Patient	Age (years)	Sex	Immunoglobulin type	Stage	Previous treatment
1	46	M	IgG kappa	3A	MP, VAD, ABMT, dexamethasone, MCNU-VMP
2	45	M	IgA kappa	3A	VAD, EDAP, PBSCT, IFNα, radiation
3	55	F	IgG kappa	3A	VAD, PBSCT, dexamethasone, IFNα
4	57	F	BJP lamda	3B	L-PAM + ADR + dexamethasone, VAD
5	58	M	IgG kappa	3A	VAD, VCAP
6	58	M	IgA lamda	3A	VAD, dexamethasone, MP, VP-16
7	70	F	IgG kappa	3A	Dexamethasone, VCAP
8	70	F	IgG kappa	3A	Dexamethasone, melphalan, VCAP
9	63	F	IgG kappa	3A	MP, VAD
10	59	M	IgA kappa	3A	VAD, dexamethasone, VCAP
11	55	F	IgA lamda	3A	Dexamethasone, VCAP, VCAD

AMBT, autologous blood and marrow transplantation; EDAP, etoposide, cisplatinum, dexamethasone and ara-C; F, female; Ig, immunoglobulin; L-PAM + ADR, melphalan and adriamycin; M, male; MCNU, ranimustine; MP, melphalan and prednisolone; PBSCT, peripheral blood stem cell transplant; VAD, vincristine, doxorubicin and dexamethasone; VCAD, vincristine, cyclophosphamide, adriamycin and dexamethasone; VCAP, vincristine, cyclophosphamide, doxorubicin and prednisolone; VMP, vincristine, melphalan and prednisolone.

than 25% of all nucleated bone marrow cells, 'severe' if the myeloma cells accounted for over 75% of all nucleated bone marrow cells, and 'moderate' if the invasion was between 'mild' and 'severe'.

Estimation of VEGF and FGF-2 concentrations in MM patients' plasma

Plasma samples were collected from 10 patients with refractory myeloma (cases 2–11) before and after 2–4 weeks of thalidomide administration. FGF-2 and VEGF were measured with an enzyme-linked immunosorbent assay (ELISA) system (R&D Systems, Minneapolis, MN, USA). Briefly, the plasma was collected and, after adding EDTA as an anticoagulant, was stored at –80°C. Patients' samples were applied to microtiterplates coated with a specific monoclonal antibody, and they were incubated at room temperature for 2 h. The plates were then washed three times and, after adding peroxidase-conjugated secondary polyclonal antibodies specific for the primary antibodies to the wells, they were incubated at room temperature for 2 h. After washing the wells, a substrate solution was added and the intensity of the blue color products was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The limit of detection of FGF-2 and VEGF in plasma was 1 pg/mL and 15.6 pg/mL, respectively. The FGF-2 and VEGF concentrations were considered elevated if they exceeded the highest value in the healthy control group. The cutoff values of FGF-2 and VEGF were 7.67 pg/mL and 38.3 pg/mL, respectively.

Thalidomide treatment and assessment of therapeutic effectiveness

Thalidomide was supplied by Sociedade Farmaceutic Brasifa Ltda. (Rio de Janeiro, Brazil) and administered *per os* at a dose of 200 mg/day for 7 days. No chemotherapeutic agents, including steroids, or radiotherapy was administered during thalidomide treatment. Certain supportive therapies, including blood transfusion, granulocyte colony-stimulating factor (G-CSF), supplemental gamma globulin, and/or pamidronate disodium administration were permitted concomitantly. If no serious side-effects were observed during the first week, the dose of thalidomide was increased to 400 mg and continued as a maintenance dose. When side-effects, such as granulocytopenia, were observed in patients after administering the increased dose of thalidomide, the dose was decreased to 200 mg/day. The effectiveness of thalidomide was evaluated by classifying the patients according to their response into the following groups: a responsive group, with a more than 30% decrease in serum M-protein or daily urine Bence Jones protein concentration sustained for at least 4 weeks; a stable group, with a less than 25% change in M-protein level; and a progressive group, with a more than 30% increase in M-protein level.

RESULTS

Microvessel density of the bone marrow of untreated patients

The MVD of the bone marrow of the 51 patients with MM or MGUS and the six control patients with non-hematological disorders is shown in Fig. 1. The typical histological findings of the bone marrow observed while counting microvessels are shown in Fig. 2. The MVD of the bone marrow in the control group was 20.4–70.9 vessels/mm^2 ($n = 6$, 43.5 ± 20.3 vessels/mm^2). The MVD of the bone marrow of MGUS patients varied widely, from 38.9 to 133.3 vessels/mm^2 ($n = 9$, 78.2 ± 30.1 vessels/mm^2), and the range in the MM patients was 38.9–264.8 vessels/mm^2 ($n = 42$, 111.1 ± 60.1 vessels/mm^2). Thus, the MVD of the BM of the MM patients was

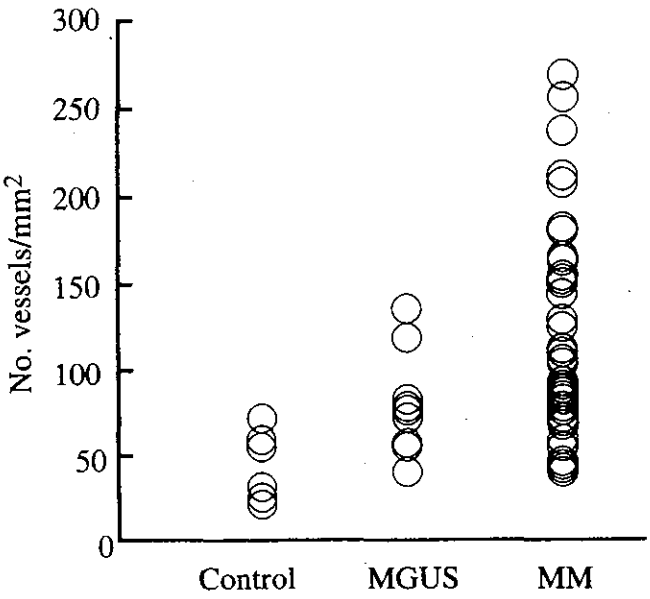

Figure 1 Microvessel density (MVD) of the bone marrow of untreated multiple myeloma (MM) patients. The MVD of the bone marrow of 51 patients and six non-hematological control patients are shown. The MVD was calculated as the number of CD34-positive microvessels observed in five fields (area of each field: 1.08 mm^2) in the bone marrow under the 80x lens of a light microscope. The MVD was 20.4–70.9 vessels/mm^2 in the control group ($n = 6$), 38.9–133.3 vessels/mm^2 in the monoclonal gammopathy of undetermined significance (MGUS) patients ($n = 9$), and 38.9–264.8 vessels/mm^2 in the MM patients ($n = 42$).

higher than in the controls; however, the increases in MVD in the MM and MGUS patients' bone marrow were not statistically significant compared with the controls.

Representative histological findings in the bone marrow of an untreated MM patient and normal bone marrow are shown in Fig. 2. Myeloma cell invasion in the bone marrow was graded by examination of HE-stained sections (Fig. 2a–d) and sections immunohistochemically stained with antiplasma cell antibody VS38c (Fig. 2e–h). There were no differences between the numbers of CD34-positive microvessels in the bone marrow with 'mild' invasion by myeloma cells and in normal bone marrow; however, there were clear increases in the number of microvessels in the bone marrow with a 'moderate' or 'severe' invasion by myeloma cells (Fig. 1). The marrow space of the bone marrow with 'severe' invasion had been replaced by numerous infiltrating myeloma cells accompanied by fibrosis. There was a marked increase in the number of microvessels in the bone marrow with a 'severe' invasion compared with normal bone marrow.

To evaluate the tendency toward increased angiogenesis in the bone marrow of MM patients statistically, we examined the MVD values of bone marrow with mild, moderate, and severe invasion by myeloma cells, and normal bone marrow. The results showed that MVD increased with the grade of myeloma cell invasion (Fig. 3). The mean MVD of normal bone marrow and the mild invasion cases was 43.5 and 79.5 vessels/mm^2, respectively, as opposed to 113.1 and 167.4 vessels/mm^2 in the moderate and severe invasion cases, respectively. The MVD of bone marrow in the moderate invasion cases and the severe invasion cases was significantly higher than in normal bone marrow ($P < 0.05$ and $P < 0.005$, respectively).

Thalidomide treatment and angiogenesis in bone marrow

Eleven refractory cases (six females and five males) that had been treated with traditional chemotherapy were treated with thalidomide (Table 2). Figure 4a shows the concentrations of M-protein in serum (cases 1–3, 5–11) and urine (case 4) before and after thalidomide treatment. Cases in which the M-protein concentration in serum or urine decreased to below 70% after thalidomide treatment were defined as 'responsive' cases. Cases with over 130% of the initial M-protein concentration after treatment were defined as 'progressive' cases. Cases with 70–130% of the initial concentration of M-protein after treatment were defined as 'stable' cases. Of the 11 cases treated with thalidomide, seven (64%) were 'responsive', three were 'stable', and one was 'progressive'. The urine concentration of M-protein in case 4 was 6010 mg/dL before treatment with thalidomide, and decreased to 1050 mg/dL after 4 weeks of treatment. In case 1, the concentration of serum M-protein changed from 5940 mg/dL to 2394 mg/dL after administration of thalidomide. In the 'responsive' cases, the grade of plasma cell invasion in the bone marrow was lower or unchanged after treatment. In the 'stable' and 'progressive' group, the grade of plasma cell invasion was unchanged or had increased after thalidomide treatment. There was an obvious decrease in the degree of bone marrow invasion in some of the patients in whom thalidomide treatment was effective.

Figure 4b shows the MVD of the bone marrow before and after thalidomide therapy. In the seven 'responsive' cases, MVD decreased in three cases (cases 6, 9 and 11) and increased in three cases (cases 1, 4, and 5) and was stable in one case (case 10). The MVD of case 10 was lower than the other cases before and after the treatment with thalidomide. The MVD increased in all of the 'stable' cases (cases 3, 7 and 8), but decreased in the 'progressive' case (case 2). No tendency toward a correlation between the effectiveness of thalidomide and the changes in the MVD of the bone marrow after thalidomide treatment was found.

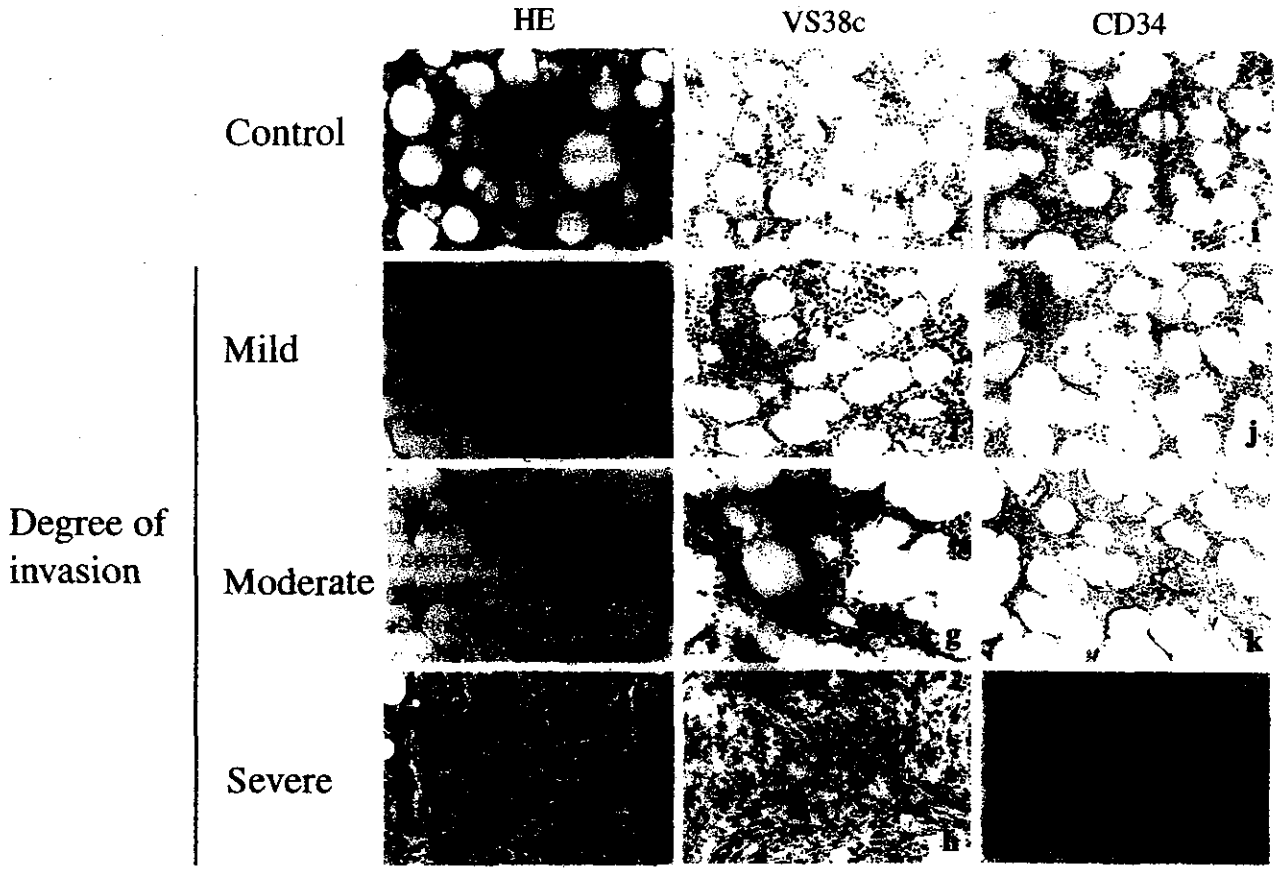

Figure 2 Grading of myeloma cell invasion and angiogenesis in the bone marrow of untreated multiple myeloma (MM) patients. Representative histologies of the bone marrow of an untreated MM patient and normal bone marrow are shown. Myeloma cell invasion in the bone marrow was graded by examining (**a,b,c,d**) sections stained with hematoxylin–eosin (HE) and (**e,f,g,h**) sections immunohistochemically stained with antiplasma cell antibody, VS38c. (**i,j,k,l**) Immunohistochemical staining with anti-CD34 antibody was performed to visualize the blood vessels. The number of microvessels in the bone marrow with 'moderate' or 'severe' myeloma cell invasion was obviously increased (**k,l**). In the bone marrow with 'severe' invasion, the marrow space was replaced by numerous infiltrating myeloma cells and accompanied by a fibrosis. There was a marked increase in the number of microvessels in the bone marrow with 'severe' invasion, compared with normal marrow (**i**).

Plasma FGF-2 and VEGF concentrations after thalidomide treatment

The plasma concentrations of angiogenic factors, FGF-2 (bFGF) and VEGF, were measured by ELISA in 10 patients treated with thalidomide. Before thalidomide treatment, the plasma FGF-2 concentrations in all of the cases in which FGF-2 was detectable (nine cases) exceeded the normal range (FGF-2 was not detectable in the plasma in case 4) (Fig. 5a). The highest plasma value was 278 pg/dL, in case 6. After 2–4 weeks of thalidomide treatment, the FGF-2 concentration had decreased in all nine cases, including the progressive case (case 2) with an increased serum M-protein concentration and the cases with increased MVD of the bone marrow (cases 3,5,7 and 8) after treatment with thalidomide.

Plasma VEGF concentrations were also determined by ELISA (Fig. 5b). Before the thalidomide therapy, an increased VEGF concentration of plasma was seen in nine cases, and the highest value was 208 pg/dL (case 5). In these nine cases, after 2–4 weeks of thalidomide treatment, a decrease of VEGF concentration was observed in eight cases, and the plasma VEGF concentration in six of these eight cases was within the normal range. These decreases in plasma FGF-2 or VEGF concentration may also have been caused by thalidomide.

Representative histological sections of bone marrow before and after thalidomide treatment in case 9, a 'responsive' case, are shown in Fig. 6. The myeloma cell invasion in the bone marrow had improved after treatment, and the number of CD34-positive microvessels had decreased. Immunohistochemistry with anti-FGF-2 antibody revealed that the cytoplasm of many hematopoietic cells, including myeloma cells, was positive (Fig. 6). There were no significant changes in the FGF-2 staining pattern after thalidomide treatment, despite the decreased concentration of FGF-2 after treat-

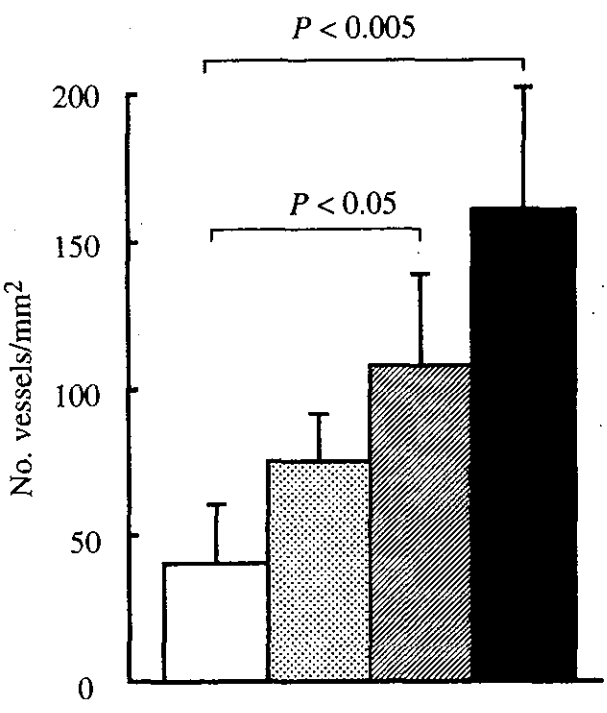

Figure 3 Increased microvessel density (MVD) in the bone marrow of multiple myeloma (MM) patients. The MVD was measured in the bone marrow with mild, moderate and severe invasion of myeloma cells and in the normal bone marrow in order to statistically evaluate the tendency toward increased angiogenesis in the bone marrow of MM patients. The MVD increased with the grade of myeloma cell invasion. The mean MVD in normal bone marrow and mild invasion cases was 43.5 and 79.5 vessels/mm^2, respectively, as opposed to 113.1 and 167.4 vessels/mm^2 in the moderate and severe invasion cases, respectively. The increased MVD in the moderate and severe invasion cases were significant compared with normal bone marrow ($P < 0.05$ and $P < 0.005$, respectively). (□), Control ($n = 6$); (▨), mild invasion ($n = 14$); (▨), moderate invasion ($n = 28$); and (■) severe invasion ($n = 9$).

ment. In contrast, FGF Receptor 1 was also widely expressed by hematopoietic cells, including myeloma cells (Fig. 6). No alterations in FGF Receptor 1 expression were seen after treatment.

DISCUSSION

The angiogenesis in solid tumors has been thought to play a role in tumor growth, invasion and metastasis.[31] In hematological malignancies, the tumor cells invade the bone marrow space and proliferate, and they replace the normal hematopoietic area because the bone marrow space is limited by the surrounding trabeculae of bone. The results of the present study showed that the MVD of the bone marrow of MM patients was higher than in the healthy controls, and greater angiogenesis was observed in MM patients with higher grades of myeloma cell invasion of the bone marrow. These findings suggest that the relationship between angiogenesis and development of MM is similar to their relationship in solid tumors.[31] Although it is not yet clear whether angiogenesis is indispensable to the pathogenesis of the disease, if angiogenesis is necessary for the development of MM, inhibition of angiogenesis may be a useful means of treatment.[19]

Vacca *et al.* reported a positive correlation between angiogenesis and the disease activity of MM.[32] Furthermore, the increased angiogenesis in bone marrow and increased levels of stimulators of angiogenesis, including FGF-2, VEGF and HGF, have recently been reported in human leukemia patients.[6,33] This has led to discussion of the possibility of antiangiogenic therapy for hematological malignancies. Thalidomide has been used to treat some MM patients as a new therapy for MM because it has an antiangiogenic effect. By using a rabbit cornea micropocket assay, D'Amato *et al.* has shown that thalidomide inhibits FGF-2-induced angiogenesis.[24] Thalidomide has also been reported to suppress production of tumor necrosis factor alpha by macrophages and to stimulate production of interleukin-2, -4, -10, and IFNγ.[34–37] These immunomodulating functions of thalidomide may contribute to the suppression of the survival and/or growth of myeloma cells. Tosi *et al.* have reported that thalidomide may suppress the progression of MM via impaired production of VEGF by myeloma cells.[38]

In the present study, we have shown that thalidomide may be effective for impairing tumor angiogenesis in the bone marrow of MM patients whose disease is refractory to conventional chemotherapy and that thalidomide reduced the plasma FGF-2 and VEGF level in almost all refractory MM cases. The cause of the decreased level of FGF-2 after thalidomide administration is unknown.

In the present study, we considered the relationship between plasma angiogenic factor (FGF-2, VEGF) level and MVD in the bone marrow of the refractory MM cases treated with thalidomide. Increased MVD was observed in the bone marrow after administration of thalidomide in some patients despite the depressed disease activity and the decreased concentrations of FGF-2 and VEGF. This discrepancy may be caused by the assessment procedure for tumor angiogenesis in the bone marrow. Singhal *et al.* also could not demonstrate a clear relationship between the bone marrow MVD and the response to the treatment with thalidomide in myeloma.[39] Hlatky *et al.* has stated that the efficacy of antiangiogenic agents cannot be simply visualized by alterations in MVD during treatment because the MVD may be outward and influenced by shrinkage, necrosis or apoptosis of the tumor.[40]

Immunohistochemistry showed that many of the hematopoietic cells and myeloma cells produced the FGF-2 protein. The FGF-2 receptors are also expressed in hematopoietic cells and myeloma cells. These results may show an auto-

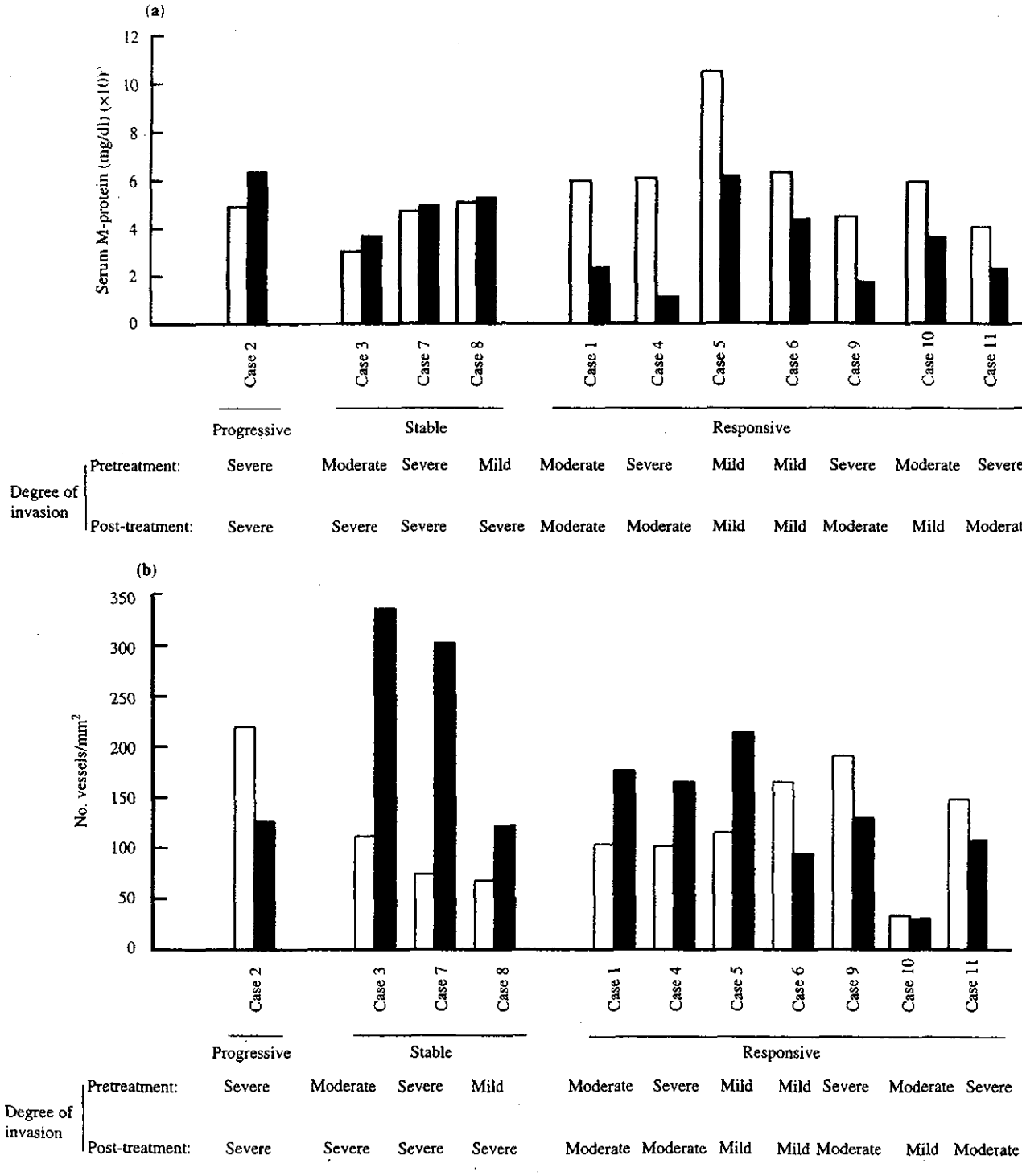

Figure 4 Effect of thalidomide treatment and change of microvessel density (MVD) of the bone marrow. (**a**) Changes in serum M-protein after thalidomide treatment. The changes in concentration of M-protein in serum (cases 1–3, 5–11) and urine (case 4) after thalidomide administration are shown. The cases were classified into three groups according to the effect of thalidomide: 'responsive'; 'stable'; and 'progressive', based on the degree of change in M-protein concentration. There were seven 'responsive' cases, three 'stable' cases, and one 'progressive' case. (**b**) Changes in MVD of the bone marrow after thalidomide treatment. The MVD of the bone marrow before and after thalidomide treatment are shown. In the 'responsive' group, MVD decreased in four cases (cases 6, 9, 10 and 11) and increased in three cases (cases 1, 4 and 5). The MVD increased in all of the 'stable' cases (cases 3, 7 and 8), but decreased in the 'progressive' case (case 2) despite the increased disease activity. (□), Pretreatment of thalidomide; (■), post-treatment of thalidomide.

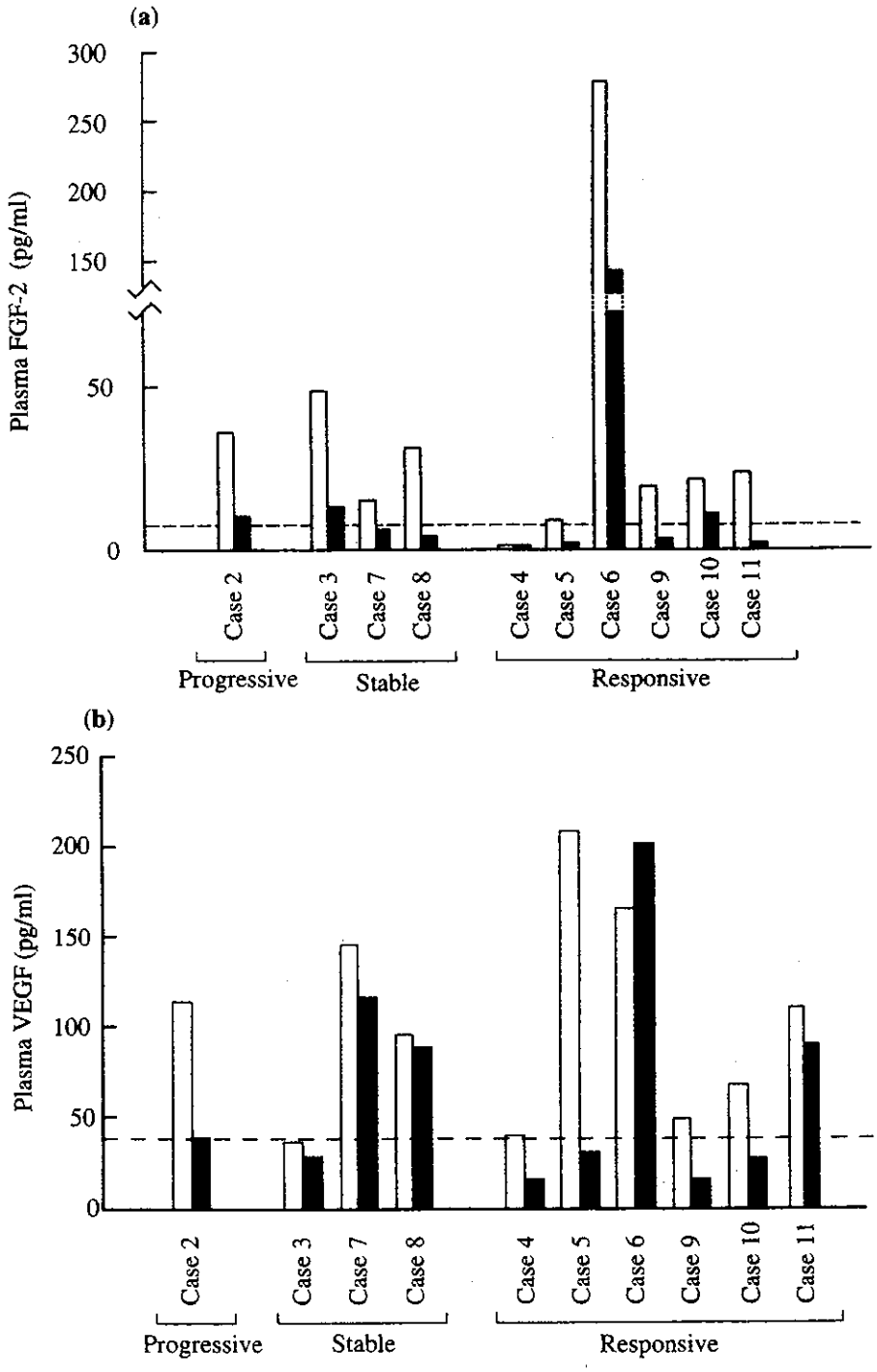

Figure 5 Plasma fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) concentrations of multiple myeloma (MM) patients before and after thalidomide treatment. The concentrations of FGF-2 and VEGF were determined in 10 of the 11 cases by enzyme-linked immunosorbent assay. (**a**) Plasma FGF-2 concentrations. Before thalidomide treatment, the FGF-2 concentration in all cases in which FGF-2 was detectable (9 cases) was higher than in the healthy subjects. The highest value was 278 pg/dL, in case 8. The FGF-2 concentration decreased in all cases after thalidomide treatment, and in five cases (cases 5, 7, 8, 9 and 11) it decreased to below the upper limit in the healthy subjects. FGF-2 was not detectable in case 4. (**b**) Plasma VEGF concentration. Before thalidomide administration, the VEGF concentration in eight cases (cases 2, 5, 6, 7, 8, 9, 10 and 11) was higher than in the healthy subjects. The highest value was 208 pg/dL, in case 5. After thalidomide treatment, a decrease in VEGF concentration was observed in eight cases, and in five cases it decreased to below the upper limit in the healthy subjects. (---), Average of plasma cytokine; (□), pretreatment of thalidomide; (■), post-treatment of thalidomide.

crine loop in which the FGF-2 produced by myeloma cells affects the growth or survival of the myeloma cell. The high concentration of FGF-2 in the patients' plasma and the possible existence of an FGF-2 autocrine loop in myeloma cells suggests the possibility of using anti-FGF-2 and/or anti-FGF-2 receptor antibody as a new form of therapy.[41] The bone marrow specimens of the MM patients treated with thalidomide also stained with anti-VEGF antibody. However, there was no positive finding in all specimens because of the manipulation of decalcification.

In the present study, we showed that seven out of 11 refractory MM cases were responsive, three cases were stable, and one case was progressive for the treatment of thalidomide. In the three stable cases, two patients were of advanced age (cases 7 and 8). The patient in the progressive case (case 2) was previously treated with irradiation and

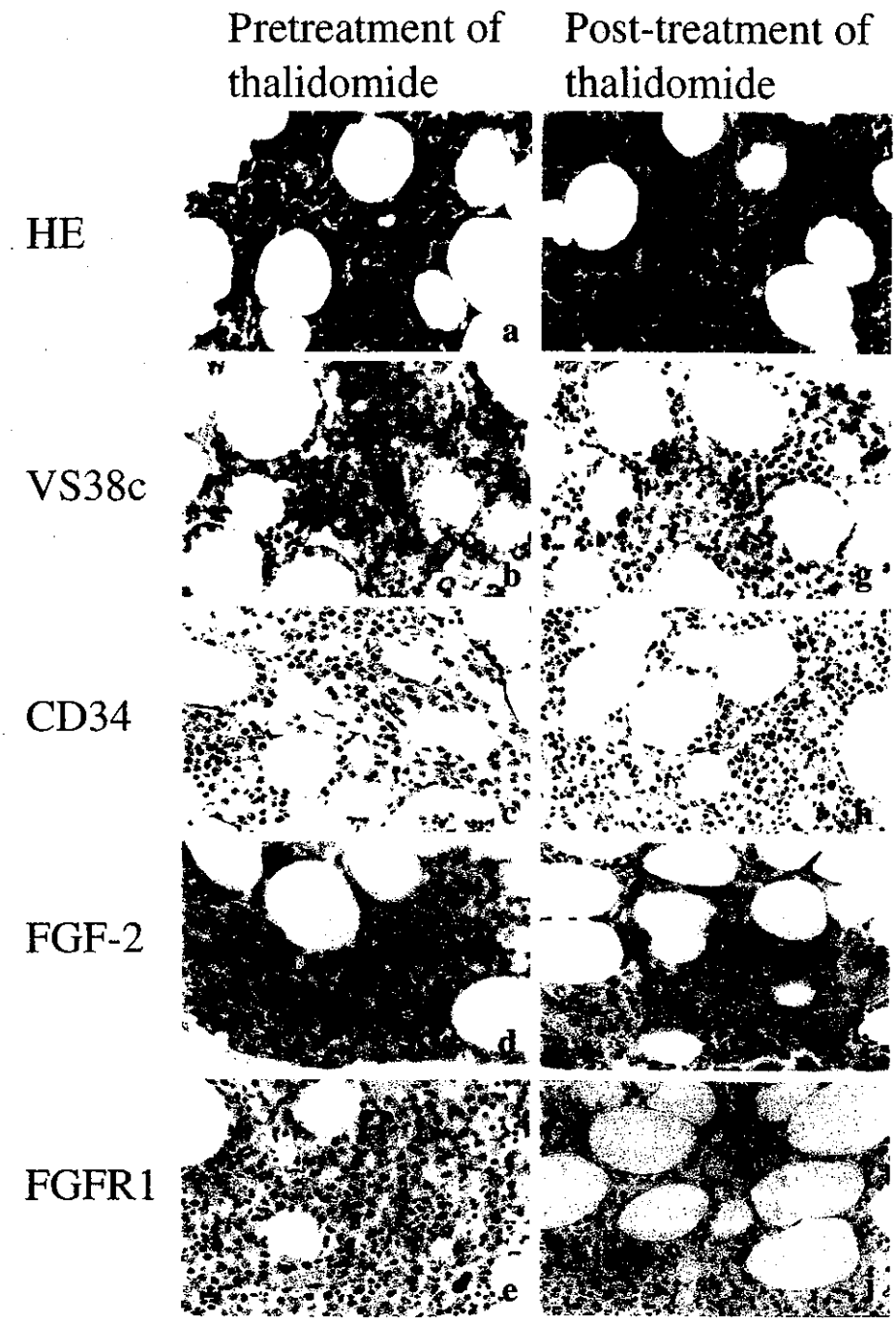

Figure 6 Histologies of the bone marrow before and after thalidomide treatment. Representative histologies of the bone marrow in case 9, a 'responsive' case, before and after thalidomide treatment are shown. (**a**,**b**,**f**,**g**) The myeloma cell invasion of the bone marrow improved after treatment. (**c**,**h**) The number of CD34-positive microvessels also decreased after treatment. Expression of fibroblast growth factor (FGF-2) was observed in the cytoplasm of hematopoietic cells and myeloma cells. (**d**,**i**) There was no significant change in the FGF-2 staining pattern after thalidomide treatment. (**e**,**j**) FGF Receptor 1 was also widely expressed in hematopoietic cells and myeloma cells, but there were no changes in its expression after treatment.

showed severe anemia before the treatment of thalidomide. Therefore, the response to the thalidomide may be influenced by age and previous therapies.

ACKNOWLEDGMENTS

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【総論：ウイルムス腫瘍】

Wilms' Tumor

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Key words
Wilms tumor, Embryonal tumor,
Congenital anoma ly

はじめに

神経芽腫，ウイルムス腫瘍，肝芽腫，胚細胞腫瘍に代表される胎児性腫瘍は，主に乳幼児期に発生する特有な腫瘍群である。これら胎児性腫瘍は器官形成途上の母細胞から発生し，腫瘍細胞が発生母地の有する分化・成熟能を潜在的に有している。一方，腫瘍発生機構においては遺伝子異常が直接的な要因であり時に，環境の付加的要因により修飾されることが明らかになっている。このような発癌に関連する遺伝子の多くが，正常組織での細胞の増殖や分化または器官形成に強く関わっていることが，明らかにされつつある。ウイルムス腫瘍は胎児性腫瘍の代表的な腫瘍である。本腫瘍はネフロンの形成に与る後腎組織の細胞を発生母地とし，形態学的にも後腎芽細胞が尿細管，糸球体に分化する過程を模倣している。

本腫瘍の歴史は古く，1814年にイギリスの臨床医であったRanceが17ヶ月男児の急激に増大した腎腫瘍を，また1828年には同じく小児の腎腫瘍をGardnerが記載している。詳細な臨床的記載を初めて行ったのはVan der Bylで，巨大な腎腫瘍として解剖まで行っている。このような報告から本腫瘍が患児に死をもたらす腫瘍であり，治療の必要性が認識されるようになった。1877年にこの腫瘍に対して，最初の腎摘術が行われた。その時期には，本腫瘍はembryonal sarcoma, sarcoma of the kidney, addenomyosarcomaなどと呼ばれていた。病理学的所見の最初の記述は，1872年，Eberthによって行われた。彼は17ヶ月女児の両側性腫瘍について検索したが，本腫瘍が極めて豊富な横紋筋成分を含んでいることを認めている。胎児性横紋筋腫性腎芽腫(fetal rhabdomyomatous nephrobalastoma, FRN)であったと考えられる。彼は本腫瘍の由来をWolffian bodyであると述べている。その後，Cohnheimが横紋筋細胞とともに他の間葉系細胞の未熟な細胞や上皮様細胞が混じった腫瘍であることを記載した。このような初期の病理学的記載から判断すると，当時は発生途上の腎生殖隆起および筋節と密接に関連して発生する腫瘍であると考えられていたのであろう。このような報告にもあるように，本腫瘍の組織像は極めて多彩である。また，後述されているように多種の腫瘍が含まれており，どこまでを真のウイルムス腫瘍に含めるかは議論の多いところである。

1899年にドイツの医学者Max Wilmsが彼の医学書に本腫瘍が記載したことによって，以後ウイルムス腫瘍(Wilms' tumor)と呼ばれるようになった。本腫瘍は疫学的にも極めて興味深い。すなわち白人種に多く，日本人を含めて「有色人種」に少ない。因みに，米国の統計では16歳以下の10,000人に1人の割合で発生し，年間460名内外の患者が発生する。すなわち，小児がんの6%を占めている。また，白人種は有色人種の3倍の頻度で多い。わが国の正確な発生数は明らかではないが，年間100名以下の発生と推定される。このような人種による発生頻度の差異は本腫瘍の発生や生物学的特異性を明らかにする上で，重要な所見である。本腫瘍の発生母地や腫瘍発生機序に関しては，ウイルムス腫瘍の歴史を反映して多くの説が提唱されてきた。その詳細は，各論を参照して頂きたい。

一方，発生母地に関しては組織像の類似性から腎発生途上に生じる後腎芽細胞に由来するという見解

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が一般的である。すなわちウイルムス腫瘍は後腎組織由来の腫瘍で，同組織を構成する後腎芽細胞が胎生30週以前に尿管芽に誘導されて糸球体や尿細管に分化する途上の細胞が腫瘍化したものと考えられる。従って，腫瘍組織は腎臓の皮質構造を模倣した像を示す。腫瘍性の後腎芽細胞(blastemal cell)は多分化能を有しており，糸球体様ならびに尿細管上皮に分化した上皮様細胞(epithelial cell)と同時に横紋筋，平滑筋，軟骨，脂肪などの間葉系細胞にも分化し，腫瘍構成成分の一部となる。このような典型的な腫瘍は古典的ウイルムス腫瘍（腎芽腫，nephroblastoma）と称されている。

また，腫瘍の前駆病変（または前がん病変）として腎芽腫症（nephroblastomatosis またはnephrogenic rest）が存在する。本病変には腎小葉内に存在する葉内腎芽腫症(intralobular nephroblastomatosis)および辺葉腎芽腫症(perilobular nephroblastomatosis)がある。それぞれの組織像およびこれらから発生する腫瘍の組織像も異なる。詳細は各論にゆずる。さて，ヒトのウイルムス腫瘍に類似した腫瘍は，多くの動物種に自然発症あるいは実験的に作成できることが知られている。特に，自然発症する動物は広汎で，ほ乳類をはじめとしてウナギ，マスなどの魚類にも生じる。

ウイルムス腫瘍の特異性は，家族内発生が多いこと，患児の胚細胞系列に染色体異常を伴い奇形をしばしば合併することにある。ウイルムス腫瘍と染色体異常，奇形の合併は古くから知られていた。しかも，染色体異常と奇形の発生に強い相関が認められることも，特記すべきことである。単独奇形としては，無虹彩症，停留精巣，尿道下裂などの生殖泌尿器系奇形が主なものである。奇形症候群としては無虹彩症・生殖泌尿器奇形・精神発達遅滞を伴うWAGR症候群（ウイルムス腫瘍，無虹彩症，genitourinary malformation, mental retardation syndrome），早期に発症し，急速に進行する腎不全，性分化異常およびウイルムス腫瘍を合併するDenys-Drash症候群（Drash症候群）が知られている。

この種の単独奇形ないしは奇形症候群では11p13領域の欠失が認められることから，この領域からウイルムス腫瘍に関わるがん抑制遺伝子の一つであるWT1遺伝子(*WT1*)が単離され，ウイルムス腫瘍の発生ばかりでなく腎および生殖器の器官形成，性の分化に重要な機能を有することが明らかにされつつある。この点は後に詳述する。一方，片側肥大などの単独奇形，または奇形症候群として巨舌，片側肥大症，臍ヘルニア，副腎の巨細胞を伴うウイルムス腫瘍もあり，Beckwith-Wiedemann症候群と呼ばれている。これらは11p15領域に存在する一連の刷り込み遺伝子(imprinting genes)が関与していることが明らかとなった。この詳細も各論で解説されている。また，家族性ウイルムス腫瘍の発生は欧米に多く，わが国には少ない。1969年に始まった米国のウイルムス腫瘍多施設共同研究であるNational Wilms Tumor Study (NWTS)の集計によると，少なくとも40家系以上が報告されている。それらの症例の腫瘍および胚細胞系列(germline)の染色体を解析すると，11番染色体短腕または17番に異常がみられる。

このようにウイルムス腫瘍は胎児性腫瘍の中でも器官形成の異常と腫瘍の発生が密接に関連した極めて興味深い腫瘍である。本腫瘍の治療に関しては，主に先に述べた米国のNWTSや欧州を中心としたInternational Society of Pediatric Tumor(SIOP)の治療法改善の努力によって，古典的ウイルムス腫瘍では平均して90%以上の5年生存率を得ている。わが国では1996年に日本ウイルムス腫瘍研究グループ(JWiTS)が発足し，NWTSのプロトコールに準じた治療法を全国に広めている。毎年40例内外の登録症例がある。JWiTSでは今までわが国で試みられることのなかった腫瘍の中央病理診断に基づいて治療法の決定を行っている。また，新鮮腫瘍組織が各施設から遺伝子検索センターに送付され，人種差のあるウイルムス腫瘍のわが国の生物学的特性を明らかにすべく解析が行われている。

本特集では器官形成と腫瘍化の接点にあるこの興味深い腫瘍の研究の現状について，詳しく取り上げた。本特集を通じて多くの研究者がウイルムス腫瘍に興味を持ち日本発の研究成果が発信されることを期待したい。

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